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Original Article

HYPOLIPIDIMIC AND ANTI-DIABETIC EFFECTS OF PUFA EXTRACTS FROM SARDINELLA LONGICEPS AND SARDINELLA FIMBRIATA ON ALLOXAN INDUCED DIABETIC MICE

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ABSTRACT

Objective: The aim of the present study was to investigate and compare the hypolipidimic and anti-diabetic effects along with Omega-3 fatty acid content of fatty acid extracts from two species of fishes, *Sardinella longiceps* and *Sardinella fimbriata*.

Methods: PUFA extracts from the two species were administered on two separate sets of alloxan-induced diabetic mice subjects and various bio-chemical parameters estimated across a period of one-month and at one week intervals. A control set without inducing diabetes and another alloxan-induced diabetic-control set without administering any PUFA extracts were also kept. Bio-chemical profiles were compared against the two control sets for estimating the effects of extracts. The same extracts were subjected to gas chromatography for its quantitative analysis of the Omega-3 fatty acids.

Results: Sets administered with PUFA extracts of both species of fishes showed significant recovery in parameters like Total Cholesterol, Triglycerides, Creatinine, LDL Cholesterol and HDL Cholesterol. Sets administered with PUFA extracts from *S. fimbriata* showed remarkably higher recovery as compared to *S. longiceps*. GC analysis showed higher concentrations of DHA in the extracts from *S. fimbriata* as compared to *S. longiceps*.

Conclusion: Higher hypocholesterolemic effect of *S. fimbriata* can be attributed to its higher DHA content while its higher hypotriglyceridimic effect may be due to the combined action of DHA and EPA. Better recovery in creatinine levels for *S.fimbriata* extract indicates a potentially greater effect for DHA in renal functioning.

Keywords: PUFA, DHA, EPA, Diabetes, *Sardinella fimbriata*, *Sardinalla longiceps*

INTRODUCTION

Diabetes mellitus is a chronic metabolic disorder with a worldwide incidence of 5% in general population with a suffering population of over 246 million and its proliferance is increasing steadily with changing life styles[1-2]. It is generally associated with complications like hypercholesterolemia, hypertriglyceridemia, atherosclerosis, coronary heart disease, renal malfunctioning and hypertension[2]. Poly-unsaturated Fatty Acids (PUFA), particularly eicosapentaenoic acid (EPA; 20:5n23) and docosahexaenoic acid (DHA; 22:6n23) present in marine sources, have been found to have healing effects against several of these complications[2-3]. Fish oil or fish oil supplemented diets, PUFA extracts from fish oils or substantially pure EPA or/and DHA have all been used in several vivo experiments. Though the utility value of fish oils in the treatment of diabetes is univocal, there is considerable disparity between results of most of these studies. Disparity subsumes in several factors; response of fish oil or EPA/DHA on normal humans and humans suffering from ailments varies considerably. Responses of PUFA extracts on the lipid profile of mice are sometimes different from that of humans[4]. It is also established that EPA and DHA has divergent effects on total cholesterol and triglycerides with exact nature of their action still unknown[5]. However, in all these studies, profiling of blood glucose, total, LDL and HDL cholesterol and triglycerides seems to be the most widely used strategy to prove beneficial or adverse effects.

Studies have also shown that Omega-3 fatty acids offer a direct or indirect reno-protective effect in diabetes patients[6-7]. Diet supplemented with Omega-3 fatty acids from plant sources is known to prevent diabetic renal injury and can even reverse kidney damage in mice subjects[8]. Hence, two additional parameters serum urea and serum creatinine were considered worth monitoring.

Among the fishes rich in PUFA, Sardines have exceptionally high concentrations of EPA and DHA[9]. *Sardinella longiceps* is known to have a high concentration of PUFA (Kamasastri et.al. 1961[10] and significantly rich in EPA and to a lesser extent in DHA (Ambasankar & Balakrishnan 2006[11]. Hypocholesterolemic effect of fish oil from *S. longiceps* has also been reported (Sen et.al. 1977[12]. However, there has not been any PUFA estimation done for the equally prolific *S. fimbriata*. The purpose of this study is to determine and compare the hypolipidimic and anti-diabetic properties of PUFA extracts from these two widely available sardines in Cochin coast obtained from the same area in its range. A comparison of their recovery profile is also attempted.

MATERIALS AND METHODS

Fish samples

Freshly caught samples of the fishes, *S. longiceps* and *S. fimbriata*, were collected from the Kaalamukku landing centre (9°58'57"N, 76°14'33"E) at Kochi. Samples washed in sterile water and brought to the laboratory in an ice box within 30 minutes after collection.

Preparation of extracts

The internal organs were removed and the meat sliced. Slices were blended and centrifuged at 10,000 rpm for 15 minutes. Post centrifugation, the oil phase was separated and subjected to saponification for converting the triglycerides to free fatty acids. The fatty acid mixture was subjected to urea complexing[13] and subsequently low temperature fractional crystallization[14] performed to obtain a mixture of substantially pure PUFA.

Determination of Fatty acid composition

The composition of the PUFA in the above mixture was directly analyzed by Gas Chromatography (GC) using fatty acid methyl ester (FAME) method[15]. The fatty acids were separated by gas liquid chromatography (Thermo Trace GC Ultra) equipped with a capillary column (30m long and 0.54mm diameter) and a flame ionization detector in the presence of hydrogen and air. Nitrogen was used as the carrier gas at a flow rate of 0.8ml/min. Initial temperature was set at 70°C and was increased at a rate of 3°C/min until peak temperature of 250°C was reached. Injector and detector temperatures were kept at 260^{0}C and 275^{0}C respectively. Fatty acids separated were identified by the comparison of retention times with those obtained by the separation of a mixture of standard fatty acids. Measurement of peak areas and data processing were carried out by Thremo Chrom card software. Individual fatty acids were expressed as a percentage of total fatty acids.

Animals

Adult male albino mice (230-260 g) were obtained from the animal house of College of Veterinary and Animal Sciences, Mannuthy and housed at 22±2 in an air-conditioned chamber. Animals were maintained throughout the study at 24-28 °C, were fed a standard laboratory rat diet and water *ad libitum* and maintained in specious polypropylene cages and well ventilated animal house with 12 hrs. dark and light cycle. The experimental protocol has been approved by the animal ethics committee.

Induction of experimental diabetes

Alloxan tetra hydrate (Sigma) was dissolved in sterile distilled water. Diabetes was induced in 18 mice by intra-peritoneal injection of 185 mg/kg (5%)[16]. The mice were fasted 12hrs before and after the alloxan injection. The mice with blood glucose above 250 mg/dl, which last for at least one week, were selected for the experiment.

Study design

For the experiment, mice were randomly divided into four groups of eight numbers each and the groups were labeled I-IV as thus.

Group I : Standard Control (C)**.** Normal mice with no extra diet components were given.

Group II : Diabetic Control (DC). Mice induced with alloxan with no extra diet components were given.

Group III : *Sardinella longiceps* Group (SL). Diabetic mice orally administered with PUFA extract of *Sardinella longiceps* (1ml) daily using intra gastric tube for 28 days.

Group IV : *Sardinella fimbriata* Group (SF). Diabetic mice orally administered with PUFA extract of *Sardinella fimbriata* (1ml) daily using intra gastric tube for 28 days.

Blood preparation

The blood was collected from orbital plexus in heparinized tubes and serum was separated by immediate centrifugation of blood samples using semi ultra cooling centrifuge at 3000 rpm for 5 minutes at room temperature. This was repeated on the 0th, 7th, 14th, 21st and 28th day of the experiment from each individual mouse in the set. The following bio-chemical parameters, viz. glucose, total cholesterol, triglycerides, urea, creatinine, LDL Cholesterol, were estimated for each of the samples.

Analytical Procedure

Fasting blood glucose was estimated by glucose oxidase-peroxidase method[17]. Serum was analysed and estimated for total cholesterol[18], HDL and LDL Cholesterol[19] levels and triglycerides[20]. Urea and Creatinine levels were estimated using diagnostic reagent kits[21-22]. For each day, all parameters were expressed as a Mean ± SD across 5 samples in each set.

Statistical Procedure and Analysis

The obtained results were analyzed using pair-wise 1-way ANOVA against diabetic control and $p<0.01$ was considered as significant[23]. Recovery percentage of biological parameters were calculated using the formula

Recovery % = (Diabetic Control – Recovered Value) ÷ (Diabetic Control – Standard Control) * 100

Table 1: Variation of bio-chemical parameters for the four sets during the experimental period

	Measurement	Days				
Set		**0**	**7**	**14**	**21**	**28**
Control Set	Glucose	81.20±0.84	81.60±1.52	81.20±1.64	81.00±1.00	80.60±0.55
	Total Cholesterol	72.00±1.22	71.80±0.45	71.20±0.84	71.40±0.55	71.20±0.45
	Triglycerides	82.00±1.22	82.00±1.00	82.00±1.22	82.40±0.55	81.00±1.00
	Urea	39.20±0.84	39.40±0.55	39.80±0.45	39.00±1.00	39.20±0.45
	Creatinine	0.24±0.05	0.26±0.05	0.30±0.00	0.26±0.05	0.26±0.05
	HDL Cholesterol	39.60±0.55	39.60±0.55	39.60±0.55	39.80±0.45	39.60±0.55
	LDL Cholesterol	16.00±1.12	15.80±0.97	15.20±1.29	15.12±0.48	15.40±0.73
Diabetic Control	Glucose	322.60±2.51	319.80±2.05	319.60±1.52	319.60±0.55	320.00±1.00
Set	Total Cholesterol	181.20±0.84	181.20±0.84	180.40±0.55	180.60±0.55	180.80±0.84
	Triglycerides	251.60±1.34	252.00±1.00	251.20±1.10	251.40±1.34	250.80±0.84
	Urea	128.00±1.22	127.80±0.84	128.80±0.84	129.20±0.45	128.00±1.00
	Creatinine	3.02±0.08	3.04±0.05	3.06±0.05	3.04±0.05	2.96±0.05
	HDL Cholesterol	20.00±1.00	19.40±0.55	20.00±1.00	19.80±0.45	19.60±0.55
	LDL Cholesterol	110.88±1.39	111.40±0.49	110.16±1.73	110.52±0.86	111.04±0.95
Diabetic Set	Glucose	321.40±2.07	316.20±1.64	313.80±1.64	312.40±0.55	311.00±1.00
administered with	Total Cholesterol	182.00±1.22	171.60±1.52	161.60±1.34	142.60±1.67	129.00±1.87
PUFA extract from	Triglycerides	251.60±1.52	239.20±1.10	226.00±1.22	210.20±1.64	183.60±0.89
Sardinella	Urea	129.20±0.84	125.20±0.84	124.20±0.84	122.80±0.45	122.40±0.55
longiceps	Creatinine	2.96±0.05	2.76±0.05	2.62±0.04	2.58±0.08	2.44±0.05
	HDL Cholesterol	19.80±0.84	24.20±0.84	27.00±1.00	28.80±0.84	30.40±0.55
	LDL Cholesterol	111.88±1.96	99.56±1.44	89.40±1.12	71.76±1.44	61.88±1.23
Diabetic Set	Glucose	322.00±2.45	317.00±1.00	315.20±0.84	312.60±1.34	310.40±0.89
administered with	Total Cholesterol	182.20±0.84	169.60±0.89	157.00±1.22	130.80±2.05	117.60±2.51
PUFA extract from	Triglycerides	251.80±0.84	231.00±1.22	213.00±1.22	196.60±1.52	166.40±0.55
Sardinella fibriata	Urea	128.60±0.55	125.60±0.55	123.80±0.45	121.80±1.10	120.40±0.55
	Creatinine	2.98±0.08	2.66±0.05	2.48±0.08	2.44±0.05	2.28±0.08
	HDL Cholesterol	20.00±1.00	24.60±0.55	29.80±0.45	31.60±0.55	34.00±1.00
	LDL Cholesterol	111.84±1.61	98.80±1.47	84.60±1.08	59.88±2.89	50.32±1.62

RESULTS

Anti-diabetic effects on various parameters for the fish extracts are summarized in Table 1; values obtained for each parameter in each set across 5 samples are expressed as Mean±SD.

Effects on Serum Glucose

Serum Glucose levels quadrupled in alloxan-induced mice at the start of the experiment and remained so through out the experimental period. However, groups administered with both fish extracts showed a small decrease in levels of blood glucose (Figure Though the decrease is statistically significant ($P < 0.01$), the percentage decrease does not indicate a recovery worth assessment. Hence, both fish extracts does not largely impact the serum glucose level in diabetes induced animals.

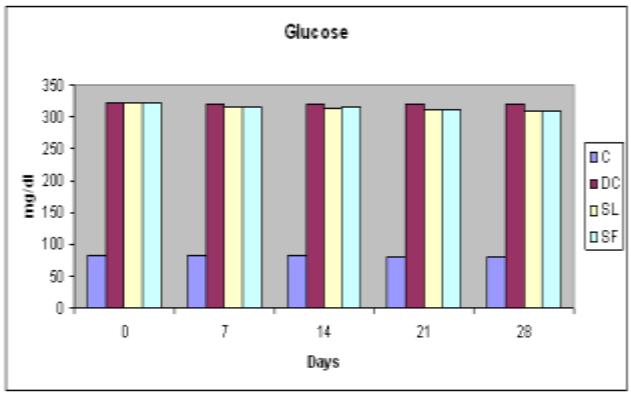

Fig.1: Glucose Variation

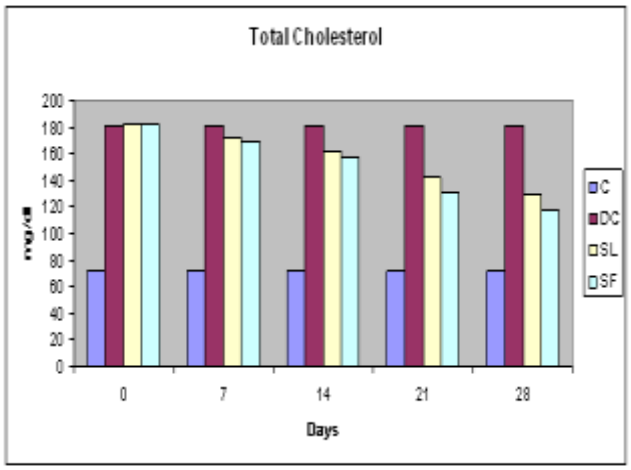

Fig.2: Total Cholesterol Variation

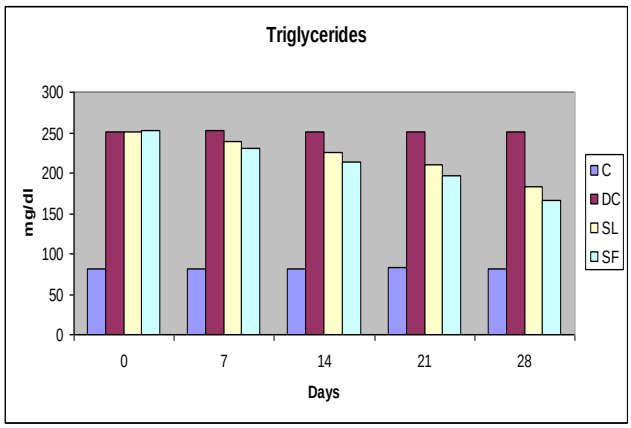

Fig.3: Triglycerides Variation

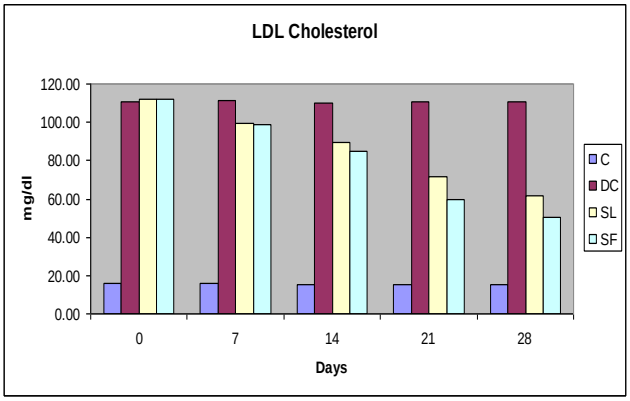

Fig.4: LDL Cholesterol Variation

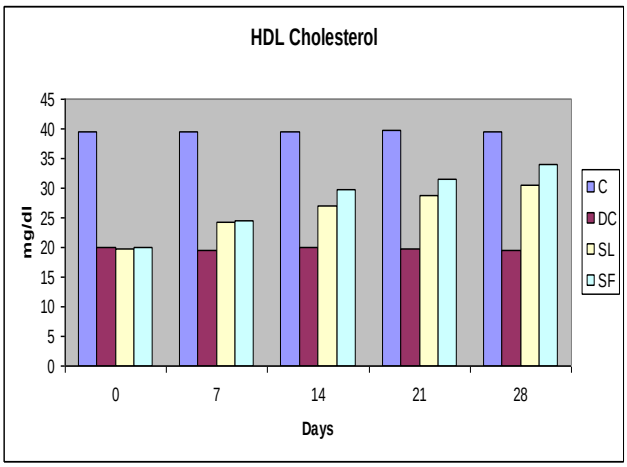

Fig.5: HDL Cholesterol Variation

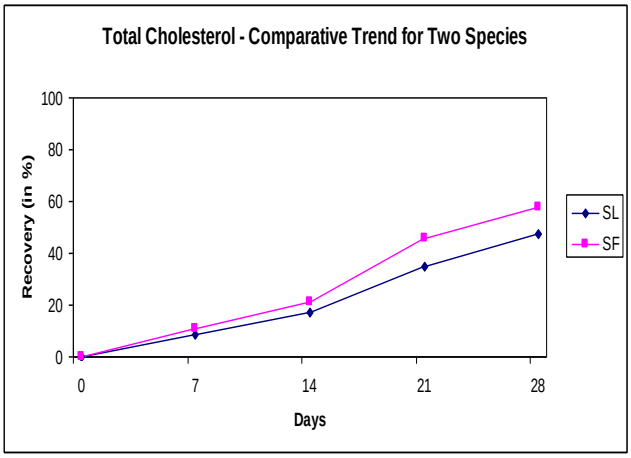

Fig. 6: Recovery of Total Cholesterol

In terms of its components, LDL Cholesterol level (Figure 4) also reduced significantly across the experiment for groups administered with extracts while HDL Cholesterol (Figure 5) which came down drastically in diabetic control improved significantly towards the end of the experiment.

Recovery plots for all four showed that sets treated with extracts from *S. fimbriata* was recovering better than the ones treated with extracts from *S. longiceps* and this became more apparent towards the end of the experiment (Figures 6,7,8,)

Effect on Cholesterol and Triglycerides

Total Cholesterol levels more than doubled and triglyceride levels tripled in alloxan-induced mice at the start of the experiment and remained so through out the experimental period. However, groups

administered with both fish extracts showed a significant recovery in total cholesterol and triglycerides (Figure 2 & 3).

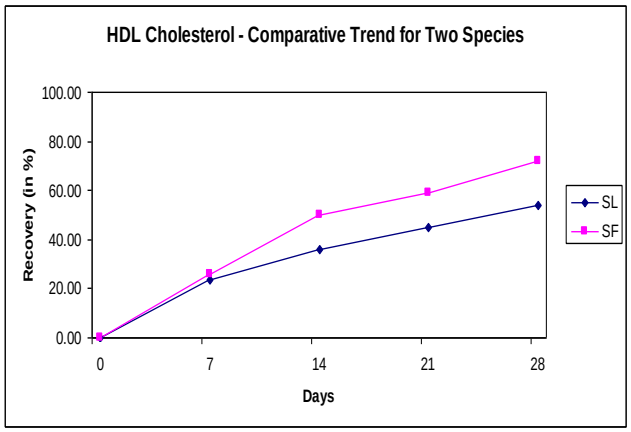

Fig.7: Recovery of HDL Cholesterol

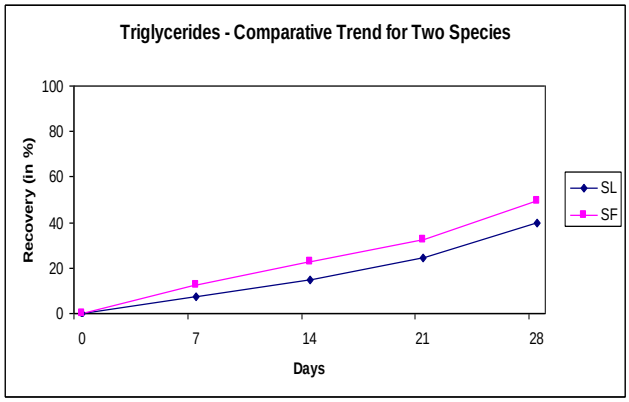

Fig. 8: Recovery of Triglycerides

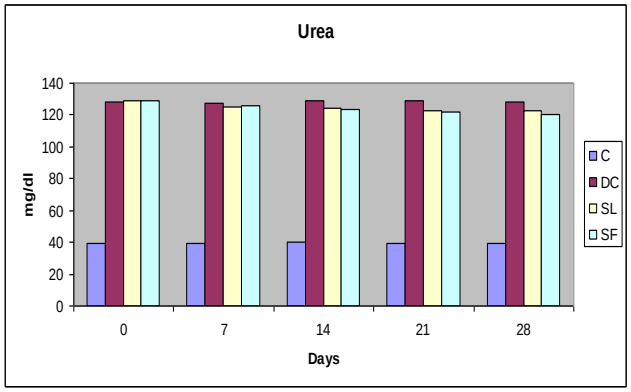

Fig.9: Urea Variation

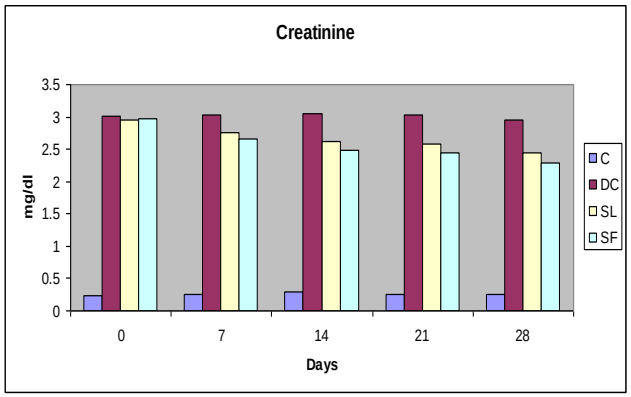

Fig.10: Creatinine Variation

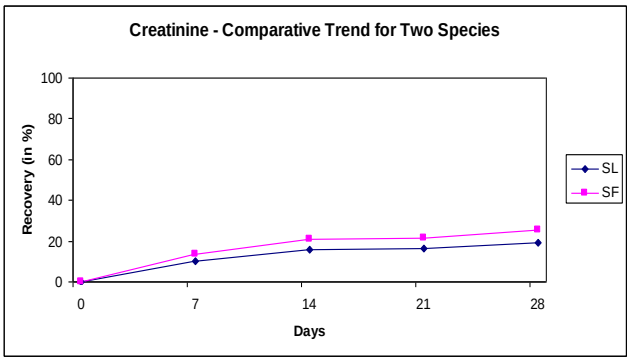

Fig.11: Recovery of Creatinine

LDL and HDL Cholesterol levels almost recovered 60% in one month after being treated with the extract of *S. fimbriata*. Triglycerides and Total Cholesterol levels recovered by 50% for this particular fish extract. Recovery was obvious in all these parameters from the first collection after drug administration (7th day) itself. A minimum of 35-40% recovery in all these parameters was observed in both species of fishes after a month and recovery curves indicated that the sets are still improving with good chances of reaching total normalcy. In summary, it can be concluded that there is considerable positive impact on Cholesterol and triglycerides of diabetic mice subjects due to the administration of these fish extracts.

Effects on Urea and Creatinine

Urea levels tripled in alloxan-induced mice while Creatinine levels shot up 12 times after inducing alloxan into the mice. The levels remained thus through out the experiment. Urea levels in sets administered with fish extracts showed a small but statistically significant ($P < 0.01$) improvement (Figure 9). However, creatinine levels improved greatly right from the first collection after drug administration (7th day) but further recovery was slow (Figures 10, 11) and did not show signs of reaching full normalcy. Yet again, sets administered with *S. fimbriata* recovered marginally better as compared to that of *S. longiceps*. In summary, it can be concluded that there is an impact on renal parameters, specifically creatinine, of diabetic subjects due to the administration of these fish extracts.

GC Analysis

The PUFA extracts were analyzed by GC to identify the fatty acids present in the extract. The major compounds identified were unsaturated fatty acids ranging from C20 to C24 with a preponderance of C22:6 (DHA) and C20:5 (EPA) PUFA. GC analyses of the PUFA from the fish *S. fimbriata* showed a DHA presence of 65.82% and an EPA presence of 24.02%, an EPA-DHA Ratio of 3:8. The GC analyses of the PUFA from the fish *S. longiceps* gave a much lower DHA figure 32.52% while an EPA figure of 55.54%, an EPA-DHA Ratio of 3:2.

DISCUSSION

The purpose of this experiment was to determine effects of two sardine oil extracts on the diabetic condition of mice. Sardine extracts used are rich in Omega-3 fatty acids[11] but with different ratio of DHA and EPA as clear from GC analysis.

Hypoglycemic Effect

Omega-3 fatty acids and fish oils are not known to affect the blood glucose levels in animals or humans. In reviews on dietary and pharmaceutical applications of Omega-3 fatty acids and fish oils[2-24], there has been no major studies cited that favourably increased blood glucose levels after the administration of fish oils or Omega-3 fatty acids. In contrast, there is a reported moderate worsening of glycemia noticeable in patients with impaired glucose tolerance and diabetes with levels > 3g/day of Omega-3 fatty acids[24]. It is generally accepted that the application of fish oils and Omega-3 fatty acids in anti-diabetic pharmacology is mainly in arresting the associated disorders like hypercholesterolemia and hypertriglyceridimia[2]. This is very much in accordance with the results of current study on mice subjects where a 28 day administration of two fish oil extracts with

differing ratios of EPA and DHA did not significantly decrease the blood glucose levels with recovery percentage being a mere 2-3%.

However, in a 60-day study on low-dose streptozocin-induced diabetic mice subjects, a decrease in blood glucose level was recorded when fed with an Omega-3 enriched diet[25] extracted substantially pure free fatty acids by urea complexing from sardine oil claimed to have found this method to be more effective (52% recovery) in treating diabetes in humans as compared to the fish oil in its natural form (12%). It is unclear on why scattered studies like the above reported a hypoglycemic effect of fish oils and Omega-3 fatty acids. However, there are several studies on plant extracts and ά-linoleic acid that have a positive hypoglycemic effect[26].Hence the lack of hypoglycemic effects for Omega-3 fatty acids may perhaps restricted only to EPA and DHA. However, a more recent study reported that colon-specific delivery of DHA and EPA on mice subjects observed substantial insulin release and subsequent glucose reduction[27].

Hypolipidemic Effects

It is well known that in uncontrolled diabetes mellitus, there will be an increase in total cholesterol, triglycerides and LDL cholesterol associated with decrease in HDL cholesterol[28]. This was in accordance with the start of the experiment in current study when alloxan induced mice tested high levels of total cholesterol, triglycerides and LDL cholesterol while HDL cholesterol decreased significantly. Patients with diabetes are at increased risk of Coronary Heart Disease (CHD) and to a clustering of risk factors for CHD, including excess weight, hypertension, dyslipidemia, and unfavorable hemostatic changes. Though there has been discordant views on the effect of Omega-3 fatty acids on CHD, evidence of Omega-3 enriched diet showing a positive correlation to reduce CHD is more overwhelming than scattered evidence of no or negative correlation[29]. Dietary Omega-3 fatty acids have been shown to be effective in reducing triglycerides and increasing HDL Cholesterol in patients with diabetes[30-31]. PUFA (EPA and DHA in excess of 65%) administered on myocardial rats significantly improved the cholesterol and triglyceride levels specifically increasing the levels of HDL Cholesterol and decreasing the levels of LDL Cholesterol[32]. In the current study, Triglycerides, LDL and Total Cholesterol decreased markedly during the 28 day course of the experiment in both PUFA extracts. Levels of HDL Cholesterol showed a sustained improvement and levels went up to 50-60% of normalcy in 28 days. These results are in accordance with similar experiments with extracts or diets rich in Omega-3 fatty acids; both in animals and in humans.

There are several studies which evidenced DHA to be a comparatively stronger hypocholesterolemic n-3 fatty acid as compared to EPA. Childs et.al[33], in their experiments on normal lipidemic men with three different concentrations of EPA and DHA, concluded that LDL and total Cholesterol were significantly lower in DHA rich diets but did not get affected by diets rich in EPA rich. However, level of triglycerides decreased significantly in all diets. They also concluded that HDL concentrations are better maintained with oil rich in DHA than EPA. In vivo mice studies have also reported specifically that DHA reduced total cholesterol significantly as compared to EPA. However, these studies also established that EPA reduces triglycerides better than DHA[34-35]. In the current experiment, extracts from *S. fimbriata* fared better over the extracts from *S. longiceps* in both total cholesterol and triglycerides. *S. fimbriata* is DHA rich and this could clearly explain the effect on total cholesterol. However, the higher response to triglycerides for the same extract cannot be explained directly in terms of the relative concentrations of these n-3 fatty acids. Perhaps, the ratio of DHA and EPA in the extract also has a role to play in the recovery of triglycerides in diabetic induced mice. However, a hypotriglyceridimic effect for DHA was shown in healthy human subjects[36] and in patients with combined hyperlipidimia[37]. Another study reported a slightly better triglyceride lowering effect in humans for DHA than EPA[38]. In mildly hyperlipidemic men, it was also found that triglycerides levels come down better with DHA than EPA[39].

Prior study indicates that fish oil from *Sardinella longiceps* demonstrates a pronounced hypocholesterolemic effect but it was not clear whether the effect was due to EPA or DHA[40]. Since there has been no similar studies on DHA rich *S. fimbriata* till date, this current study gains importance as extracts from *S. fimbriata* seems more potent as a hypocholesterolemic agent and result tallies well with erstwhile studies that proved a similar effect for DHA.

Effects on Renal Functioning

Diabetes is associated with several renal disorders and abnormal levels of serum urea and serum creatinine[30]. The diabetic hyperglycemia induced by alloxan produce elevation in plasma levels of urea and creatinine in animals, which are considered significant markers of renal dysfunction. Action of chemically induced alloxan on animals is not specific to pancreas but also affects organs like kidney[41] as is also apparent from the histopathological examination of kidneys of diabetic induced mice in this study. A 30 week study on streptozotocin-induced diabetic mice demonstrated that n-3 fatty acids are superior to n-6 fatty acids in renal functioning by controlling urine albumin excretion, glumerulosclerosis and tubulointerstitial fibrosis[42]. Prior study reported that Omega-3 fatty acids improve renal functioning in patients who undergo heart and kidney transplants[43]. Urakaze et al.[44] reviews several studies done on the effect of omega-3 fatty acids on human subjects with renal disease by assessing serum creatinine among other factors and concludes that two studies reported a statistically significant improvement in serum creatinine when treated with fish oil. The current study on diabetic mice reports a recovery of 15-20% in serum creatinine over a period if 28 days, recovery peaking within 7 days and remaining more or less steady. This reduction and the corresponding positive histopathological changes in kidney cells of treated mice suggest potential utility of these fish extracts in diabetes associated complications. It is also established that normal subjects do not show any change in renal function even when given pharmacologic doses of fish oil, which is encouraging from the safety standpoint[45].

Moreover, it also known that the beneficial effects on renal function are partly dependent on an increase in EPA and DHA[43]. The mechanism involved is unknown, but experimental studies have shown that omega-3 fatty acids may increase thromboxane A3 formation, coinciding with a fall in thromboxane A2 and a significant increase in total prostacyclin levels[46]. It is also not clear whether EPA or DHA has a greater effect. In the present study, DHA rich *S. fimbriata* showed a marginally better recovery as against the EPA rich *S.longiceps* perhaps indicating that DHA has a greater role in maintaining creatinine levels and hence renal functioning.

CONCLUSION

In conclusion, widely available marine fishes like sardines serve as a rich source of DHA and EPA and is an excellent nutritional source for human subjects having hyperlipidemia and renal disorders associated with diabetes. Though there is no significant positive influence on the blood sugar levels, the positive influence on associated disorders of these compounds creates an opportunity to be used as a supplement to the main drug. Hence these natural sources have the potential to be an excellent source of pharmaceuticals that target these disorders. Fish oil extracts from Sardinella fimbriata have higher concentrations of DHA than EPA and hence seem to have greater hypolipidemic and renal effects.

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